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Identification of different specificity requirements between SGK1 and PKBα

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Abstract **NDRG1 is phosphorylated by SGK1 (but not PKB) in vivo at three residues each contained within three nonapeptide repeats. Here, we demonstrate that this nonapeptide, like the NDRG1 protein, is phosphorylated by SGK1, but not by PKBα or RSK1 in vitro. The inability of PKBα and RSK1 to phosphorylate the nonapeptide was traced to residues $n+1$, $n+2$ and $n-4$ (where n is the phosphorylation site). Changing them from Ser, Glu and Ser to Phe, Ala and Pro, respectively, transformed the nonapeptide into an excellent substrate for PKBα and RSK1. Our results identify a specific substrate for SGK1 and may facilitate detection of additional physiological substrates for this enzyme.**

Keywords: PKB; SGK; NDRG1; Protein kinase specificity

1. Introduction

The "AGC" subfamily of protein kinases comprises enzymes that play key roles in signal transduction by extracellular agonists. They include cyclic AMP and cyclic GMP-dependent protein kinases, the diacyl-glycerol-activated isoforms of protein kinase C, ribosomal S6 kinases (RSKs, S6Ks) and mitogen and stress-activated protein kinases (MSKs) that are components of MAP kinase cascades, as well as protein kinase B (PKB/Akt) and serum and glucocorticoid-induced protein kinases (SGKs) that are activated by insulin and other signals that switch on phosphatidylinositol 3-kinase.

Nearly all AGC family members phosphorylate serine and/or threonine residues that lie C-terminal to clusters of basic amino acid residues that are critical for their specificity. For example, several AGC family members, such as PKB, SGK, RSK and S6K, preferentially phosphorylate Arg–Xaa–Arg–Xaa–Xaa–Ser/Thr motifs [1]. This allows these protein kinases to target the same amino acid residues in such proteins, allowing these substrates to respond to several physiological stimuli. For example, the protein kinase glycogen synthase kinase 3 (GSK3) can be inactivated by PKB in response to insulin [2], by RSK in response to growth factors [3] or by S6K in response to amino acids [4]. Similarly, the Tuberous Sclerosis Complex (TSC) can be phosphorylated by PKB or RSK in cells, preventing it from inhibiting activation of the mammalian target of rapamycin (mTOR) [5,6].

However, it is also obvious that each of these protein kinases phosphorylate specific targets. This is evident, for example, from the phenotypes of mice that do not express these enzymes. Thus mice that do not express the β-isoform of PKB are deficient in insulin-stimulated glucose uptake into muscle and become diabetic as they age [7], whilst SGK1-deficient mice are unable to excrete sodium ions normally from the kidney, due to defects in the regulation of several ion channels [8].

We have developed a method called KESTREL (**k**inase **s**ubstrate **tr**acking and **el**ucidation) for identifying the physiological substrates of protein kinases, and have recently exploited it to identify proteins in cell extracts that are phosphorylated by PKB, but not by SGK ([9] and R. Cartlidge, G. Auld and P. Cohen, unpublished work), with which it shares 54% sequence identity in its catalytic domain. Conversely, we have also detected proteins that are phosphorylated by SGK1, but not by PKBα, and identified them as the protein products of the N-myc downstream regulated gene NDRG1 and its homologue NDRG2 [10]. These proteins are phosphorylated in vivo at the sites targeted by SGK1 in vitro, but not in mice that do not express SGK1 or in cells where SGK1 has been "knocked down" using specific siRNAs. This and other evidence indicates that NDRG1 and NDRG2 are indeed physiological substrates of SGK1, and that PKB isoforms are unable to substitute for SGK1, even when the latter is not expressed. This is surprising since the major sites on NDRG1 and NDRG2 that are phosphorylated by SGK1 are located in canonical Arg–Xaa–Arg–Xaa–Xaa–Ser/Thr sequences.

Three of the sites in NDRG1 that are targeted by SGK1 lie in a nonapeptide sequence, Arg–Ser–Arg–Ser–His–Thr–Ser–Glu–Gly, that is repeated three times near the C-terminus of the protein [10]. In this paper, we synthesised this nonapeptide and found that, similar to the NDRG1 protein [10], it is phosphorylated efficiently by SGK1, but extremely poorly by PKBα, RSK1 and S6K1 in vitro. Thus, the specific phosphorylation of NDRG1 by SGK1 in vitro can be recapitulated using the nonapeptide. By making a number of variants of this peptide, we have been able to identify the residues that prevent phosphorylation by PKBα and RSK1. This has provided new insights into the molecular basis for the distinct specificities of

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Abbreviations: GST, glutathione S-transferase; GSK3, glycogen synthase kinase 3; PKB, protein kinase B; RSK, p90 ribosomal S6 kinase; S6K, p70 S6 kinase; SGK, serum and glucocorticoid-induced kinase

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SGK1, PKBα and RSK1 in cells, which may help to pinpoint other proteins that are physiological substrates for SGK.

2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP and materials for protein purification were obtained from Amersham Biosciences (Chalfont St Giles, UK). All other chemicals were of the highest purity and purchased from Merck (Poole, UK) or Sigma–Aldrich (Poole, UK).

2.2. Protein expression and purification

All His-tagged proteins were expressed in insect Sf21 cells. His-tagged PKBα [118–480] in which Ser473 was mutated to Asp, SGK1 [60–431] in which Ser422 was mutated to Asp and S6K1 [1–421] in which Thr412 was mutated to Glu were purified by chromatography on nickel-nitrilotriacetate (Ni-NTA) agarose and then maximally activated by phosphorylation with His-tagged PDK1 [52–556]. N-terminally his-tagged full length RSK1 [1–735] was purified and then maximally activated with PDK1 and full length glutathione S-transferase (GST)-ERK2. The PDK1 bound very tightly to heparin-Sepharose [11] and was removed from all activated protein kinases by passage through this column. GST-ERK2 was removed by passage through glutathione agarose. The activated protein kinases were concentrated by repurification on Ni-NTA-agarose, dialysed against 50 mM Tris/HCl, pH 7.5, 270 mM sucrose, 150 mM NaCl, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 0.2 mM phenylmethylsulphonyl fluoride and 1 mM benzamidine and stored at −80 °C.

2.3. Synthesis of peptides

The peptide termed Sgktide (KKRNRTLSVA) was synthesised on an Applied Biosystems 431A peptide synthesiser [12] and all other peptides were synthesised using Fmoc chemistry [13] on an Applied Biosystems 9050+ or Pioneer automatic peptide synthesiser. Peptides were purified by reverse-phase HPLC on a C_{18} silica wide-pore column (15 cm × 2.1 mm) with an elution gradient of 10–50% acetonitrile containing 0.1% (v/v) trifluoroacetic acid over 40 min. Further purification, where necessary, was performed on a 25 cm × 22 mm column using the same conditions. The molecular mass was confirmed by mass spectrometry performed on a VG Quattro triple-quadrupole instrument with electrospray ionisation. Peptide synthesis reagents were purchased from Bachem (St. Helens, UK) or Calbiochem (Nottingham, UK).

2.4. Assay of protein kinases

These were assayed at 30 °C as described previously [14,15]. One Unit of PKBα, SGK1, RSK1 and S6K1 was that amount which catalysed the phosphorylation of 1 nmol of the standard substrate peptide CROSStide (GRPRTSSFAEG) in 1 min [2]. Each peptide substrate was used at the concentrations indicated in Section 3 and incubated for 7.5 min in 25 μl reaction volumes at 30 °C with 10 mM $MgCl_2$–0.1 mM [γ-^{32}P]ATP (10^6 cpm/nmol) in 50 mM Tris/HCl, pH 7.5, containing 0.05% (v/v) 2-mercaptoethanol and 1.0 U/ml of recombinant protein kinase. Incorporation of phosphate was linear with respect to time under the conditions used. Reactions were terminated by spotting on to phosphocellulose paper, followed by immersion in 75 mM phosphoric acid. All papers were then washed four times in 75 mM phosphoric acid to remove unincorporated ATP, once in acetone and then dried and ^{32}P incorporation was determined by Cerenkov counting.

3. Results and discussion

The peptide substrate that is used to assay PKB routinely, Arg–Pro–Arg–Thr–Ser–Ser–Phe–Ala–Glu–Gly (CROSStide), is similar to the sequence surrounding the residue in GSK3 that is phosphorylated by PKB in vitro and in cells. The second serine in the peptide sequence is the site of phosphorylation and conforms to the canonical sequence motif for phosphorylation by PKB or SGK [2]. This peptide was phosphorylated by PKB and SGK with K_m values of 23 and 5 μM respectively, and V_{max}/K_m values that were also similar (Table 1, peptide 1). In contrast the nonapeptide Arg–Ser–Arg–Ser–His–Thr–Ser–Glu–Gly, which is repeated three times near the C-terminus of NDRG1, was barely phosphorylated by PKBα, RSK1 or S6K1. The K_m values were far higher than 1 mM (Fig. 1A) and the V_{max} for each kinase could not therefore be determined (Table 1, peptide 2, and data not shown). Nevertheless SGK1 phosphorylated the peptide with a K_m of 72 μM and with a V_{max} that was nearly twofold higher than CROSStide (Fig. 1A, and Table 1, peptide 2). The nonapeptide is therefore a rather specific substrate for SGK1 and is hereafter termed MURRAYtide.

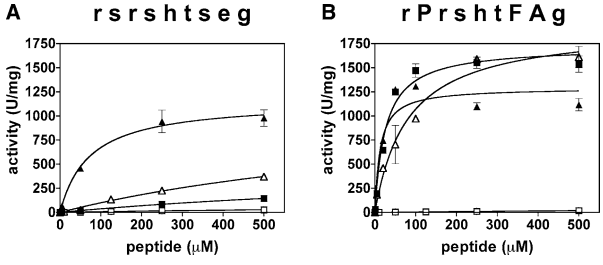

Fig. 1. Relative rates of phosphorylation of RSRSHTSEG (MURRAYtide) and RPRSHTFAG by PKBα, SGK1, RSK1 and S6K1. Peptides were phosphorylated at 30 °C for 7.5 min with 1.0 U/ml of PKBα (■), or SGK1 (▲), RSK1 (△), and S6K1 (□), which had been matched for activity against 30 μM CROSStide.

Table 1
Relative effectiveness of different peptides as substrates for PKBα, SGK1 and RSK1

	Peptide	PKB		SGK		RSK1	
		V_{max} (U/mg)	K_m (μM)	V_{max} (U/mg)	K_m (μM)	V_{max} (U/mg)	K_m (μM)
1	GRPRTS**S**FAEG	1160	23	616	5	1492	7
2	RSRSH**T**SEG	ND	>1000	1160	72	ND	>1000
3	RSRSH**T**FEG	1210	270	1280	16	573	133
4	RSRSL**T**SEG	ND	>1000	940	100	ND	>1000
5	RSRSH**T**SAG	1190	440	1300	26	ND	>1000
6	RSRSH**T**FAG	1700	43	1370	14	1237	50
7	RPRSH**T**SEG	354	220	1400	40	824	418
8	RPRSH**T**FEG	1410	110	1070	10	1102	114
9	RPRSH**T**FAG	1720	26	1290	13	1933	81

The phosphorylated residue is shown in boldface type and the substituted residues are underlined. ND, not determined.

In order to understand why MURRAYtide is such a poor substrate for PKBα, we synthesised further peptides in which one or more residues were substituted by other amino acids. Previous studies with synthetic peptide substrates have revealed that PKBα has a strong preference for a bulky hydrophobic residue at the position immediately C-terminal to the site of phosphorylation, termed $n+1$ where n is the site of phosphorylation [1]. Since this residue is serine in MURRAYtide, we initially changed it to phenylalanine. This substitution did indeed improve the ability of PKBα and RSK1 to phosphorylate the peptide (Table 1, peptide 3), but the K_m for each kinase was still at least 10-fold higher than CROSStide. Moreover, this mutation also decreased by 4–5-fold the K_m for phosphorylation by SGK1 (Table 1, peptide 3), so that the peptide was still phosphorylated far more efficiently by SGK1 than PKBα or RSK1.

None of the known physiological substrates for PKB contain a histidine residue immediately N-terminal to the site of phosphorylation ($n-1$), and we therefore wondered whether its presence in MURRAYtide might be deleterious to phosphorylation by PKBα. However, its replacement by leucine, which is reported in one study to be favoured by PKB [16], did not improve the ability of MURRAYtide to be phosphorylated by PKBα or RSK1 (Table 1, peptide 4).

We next considered the possibility that the presence of an acidic residue at position $n+2$ might be deleterious for protein kinases other than SGK1 (the glutamate residue in CROSStide is at $n+3$). The substitution of the glutamate at $n+2$ by alanine did indeed improve the ability of MURRAYtide to be phosphorylated by PKBα, and it also reduced the K_m for phosphorylation by SGK1 2–3-fold (Table 1, peptide 5). Thus, an acidic residue at this position is a negative determinant for SGK1 and PKBα. The glutamate to alanine mutation did not improve the peptide as a substrate for RSK1. However, replacing both the serine at $n+1$ with phenylalanine and the glutamate at $n+2$ with alanine greatly improved the ability of MURRAYtide to be phosphorylated by PKBα and RSK1, and this peptide was now phosphorylated by both protein kinases with a K_m value only 2–7-fold higher than CROSStide and with a similar V_{max} (Table 1, peptide 6). Nevertheless, the peptide RSRSHTFAG was still phosphorylated by SGK1 with a threefold lower K_m than PKBα, and we therefore looked for another residue(s) that might account for this difference.

A number of the physiological substrates for PKB, including FOXO1a (formerly FKHR) [17], GSK3β [2] and PDE-3B [18], possess a proline residue at $n-4$ between the two arginine residues at $n-3$ and $n-5$ that are critical for the specificity of this protein kinase, whereas this residue is serine in MURRAYtide/NDRG1 (Table 1, peptide 2). In the case of NDRG1, the presence of serine at this position is critical, since one role for SGK1 phosphorylation is to prime NDRG1 for phosphorylation by GSK3, which phosphorylates serine/threonine residues located 4-residues N-terminal to another phosphorylated serine/threonine residue [19]. Mutation of the serine at $n-4$ to proline did indeed improve the ability of MURRAYtide to be phosphorylated by PKBα and RSK1. However, for PKBα, the effect was not as marked as the mutation of glutamate to alanine at $n+2$ (Table 1, peptides 5 and 7).

After mutating all three residues, i.e. the serine at $n-4$ to proline, the serine at $n+1$ to phenylalanine and the glutamate at $n+2$ to alanine (Fig. 1B), the resulting peptide was phosphorylated by PKBα and RSK1 at least as efficiently as CROSStide (compare peptides 1 and 9 in Table 1) and with comparable kinetics to SGK1. It should be noted that after the combined mutation of these three residues, S6K1 was still unable to phosphorylate the peptide. The reason for this is unknown.

In summary, this study has introduced a specific peptide substrate for the assay of SGK1 and is likely to explain why NDRG1 is phosphorylated in vitro and in vivo by SGK1 but not by PKBα. The molecular basis for this discrimination arises from the presence of three residues within the nonapeptide repeat sequence that are negative determinants for phosphorylation by PKBα and RSK1, and which, in combination, essentially prevent the phosphorylation of NDRG1 by protein kinases other than SGK1.

Arg–Xaa–Arg–Xaa–Xaa–Ser/Thr motifs are present in many proteins. Clearly, such motifs must be located on exposed regions on the surface of proteins in order to be accessible to protein kinases and therefore not all such motifs will be phosphorylated in cells. However, the present results, in conjunction with an earlier report [20], indicates that SGK can tolerate more variation in the other amino acids surrounding this motif. One might therefore predict that SGK is likely to phosphorylate many more of these motifs in cells. This would be consistent with SGK being an immediate early gene whose synthesis increases dramatically within an hour of exposing cells to a diverse array of extracellular signals that include serum, glucocorticoids, mineralocorticoids, cellular stresses and growth factors, reviewed in [21].

In the light of our results, it may be possible to predict candidate substrates for phosphorylation by SGK, but not PKB. However, caution must be exercised in extrapolating from synthetic peptides to native protein substrates, where particular features of the three-dimensional structure or the presence of "docking sites" for particular protein kinases may sometimes modify or override the need for a "normal" recognition motif. For example, one of the phosphorylation sites (Ser319) in the transcription factor FOXO1a is followed by a serine, but is clearly phosphorylated by PKB and not by SGK in IGF-1 stimulated embryonic stem cells [22]. Similarly, although MAP kinases nearly always phosphorylate serine or threonine residues that are followed by proline, there are clearly a few physiological substrates where this "dogma" does not apply [23]. Nevertheless, information obtained with synthetic peptides is generally a useful guide and we anticipate that the present study will stimulate the identification of more substrates for SGK1 in the future.

A peptide called Sgktide (KKRNRRLSVA), has been used to assay SGK1 in vitro [24]. However, this is a misnomer because, in our assays, Sgktide is phosphorylated by PKBα and SGK1, with similar V_{max} (1540 and 1330 U/mg, respectively) and K_m values (100 and 20 μM, respectively). It is also phosphorylated by RSK1 with a V_{max} nearly twofold higher than CROSStide (data not shown). The identification of MURRAYtide as an SGK1-specific peptide substrate may lead to the adoption of this peptide as the standard substrate for the assay of SGK1 activity.

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